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From companion diagnostics to theranostics: a new avenue for Alzheimer's disease?

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Abstract

The recent literature signals a growing paradigm shift towards integrating therapeutics and diagnostics, rather than developing and deploying them separately. In this gradual move towards more effective and personalized medications, companion diagnostics are an intermediate stage. The next step may be "theranostics", in which single chemical entities are developed to deliver therapy and diagnosis simultaneously. This strategy has been successfully exploited in oncology and is now emerging as a possibility for Alzheimer's disease, where its feasibility has caught the attention of researchers from industry and academia. Medicinal chemists do not yet completely understand the nuances of theranostic action and consequently have not yet developed universally validated strategies for developing theranostic clinical applications against Alzheimer's disease. However, given the emerging indications of the potentially enormous benefits that theranostics may bring to the fight against this devastating disease, further rigorous research is warranted.

1. Introduction

In 2014, Cummings published the first-ever analysis of clinical trials of drugs to prevent, cure, or treat symptoms of Alzheimer's disease (AD).[1] "Alzheimer's Disease Drug Development Pipeline: Few Candidates, Frequent Failures" confirmed AD to be a most challenging and puzzling disease.[1] The paper highlighted that, since the approval of memantine in 2003, no new AD drug candidate

has successfully completed a phase III trial. In particular, between 2002 and 2012, 244 compounds were assessed in 413 trials for their ability to prevent, cure, or treat AD symptoms. Only memantine secured FDA approval to reach the market (0.4% success rate). At 99.6%, this overall drug failure rate is much higher for AD than for similarly serious diseases such as cancer (where it falls to 81%). Moreover, when the paper was written, there were only about 80 AD drugs in the pipeline compared to 300 cancer drugs.[1] These findings are especially alarming given the growing incidence of AD. According to Alzheimer's Disease International, there were an estimated 46.8 million people with dementia worldwide in 2015.[2] This number is expected to rise to 131.5 million in 2050, driven by global patterns of population ageing. Moreover, AD is no longer a problem for rich developed countries only. Around two-thirds of all dementia patients live in India, Brazil, and China, and that proportion is set to increase to three-quarters by 2050.[2]

The high personal, familial, societal, and financial burden of this disease demands bold and urgent action. Many countries and international organizations have thus developed national plans and analytical reports to address the problem.[3] G7 nations have reinvigorated efforts to find disease-modifying treatments or cures for dementia by 2025. The AD research budget for the USA National Institutes of Health was recently increased to nearly $1 billion per year.[4]

A similarly bold action would be a move towards personalized medicine to combat AD.[5] Early signs of this paradigm shift are already evident and could revolutionize AD healthcare. Tests that assist in diagnosis and treatment choice are becoming available for clinical practice.[6] Radiologists can now directly visualize amyloid plaques in AD patients using tests based on positron emission tomography (PET) and approved by the FDA and European Medicines Agency. Researchers are developing several companion diagnostics (CDx) to identify those patients most likely to benefit from a given therapy and to tailor dosages to a patient's specific needs. Another approach involves theranostics, wherein diagnostic and targeted therapeutic properties are combined in a single small molecule. The full clinical impact of theranostics in AD is far from clear; however, there are initial indications of success. Herein, we provide examples of CDx and theranostics from the very recent

literature to substantiate these concepts. We focus in particular on the medicinal chemistry issues that arise when designing such cutting-edge tools, and envision their potential future impact.

2. Diagnostics in AD: the status quo

While AD drug discovery has experienced a profound productivity crisis in recent years,[1] the field of diagnostics has been booming. In 2012, the FDA approved the first radioactive diagnostic to help clinicians detect causes of dementia other than AD. PET ligand **1** ([^{18}F]florbetapir)[7] is indicated for the brain imaging of cognitively impaired adults undergoing evaluation for AD and other dementias (Figure 1). Soon after, two more amyloid imaging agents were approved for clinical use, 2 ([^{18}F]flutemetamol) and **3** ([^{18}F]florbetaben).[8, 9] In addition to informing the differential diagnosis of cognitively impaired patients, these agents have emerged as a source of innovation and a valuable tool in the quest to reduce clinical dropout.[8, 9] At the clinical level, these agents create new possibilities for improving treatment and trials, by enabling patient-risk stratification, patient selection for Aβ-targeting therapies, and monitoring of patients receiving such therapies.[10] AD diagnostics could similarly impact translational medicine at a preclinical level. These agents could allow researchers to establish whether a promising drug candidate can reach and bind the biological target at an optimal concentration and sustain target engagement for the necessary period of time.[9] From a chemistry point of view, the three compounds are strictly related: **2** is a derivative of 4-(3,6-dimethyl-1,3-benzothiazol-3-ium-2-yl)-N,N-dimethylaniline chloride (thioflavin T, ThT) dye, whereas **1** and **3** are diaryl alkene analogs differing by only one atom (Figure 1). Thus, they are a clear example of *me-too products*, further corroborating the strong interest of pharmaceutical and diagnostics companies in developing imaging agents for AD. Interestingly, several of these diagnostics were developed in academic laboratories.[11] **1** was discovered by Kung's group at the University of Pennsylvania and then licensed for development by Avid, whereas **2** was developed at the University of Pittsburgh Medical Center as a [^{18}F]fluorinated derivative of the "gold standard" amyloid [^{11}C]-tracer **4** (PiB).[11]

Needless to say, the current and future effectiveness of amyloid imaging agents strictly depends on the validity of the amyloid hypothesis.[12] Whether amyloid is the correct AD biomarker is still a matter of debate. This awareness, when combined with some incoherent data and unexplained anomalies reported in amyloid-PET imaging studies using **1**-**3**, calls for a critical examination of the modality.[13] Recent findings suggest that abnormal aggregation and/or physiological malfunction of tau protein is potentially more significant for neurodegeneration, and indicate the severity of tau pathology to be an effective biomarker for the early diagnosis of AD.[14] Researchers are thus diversifying their approaches, with tau imaging agents emerging as an active area of academic and industrial investigation. This area has been comprehensively reviewed elsewhere.[15,16] We note that the in vivo proof of concept has already been achieved for 5-((1*E*,3*E*)-4-(6-[^{11}C]methylamino)pyridin-3-yl)buta-1,3-dien-1-yl)benzo[*d*]thiazol-6-ol ([^{11}C]PBB3),[17] which has been clinically validated as a PET tau imaging agent. Later, tau PET tracers 7-(6-[^{18}F]fluoropyridin-3-yl)-5H-pyrido[4,3-b]indole (T807),[18] 6-(2-[^{18}F]fluoroethoxy)-2-(4-aminophenyl)quinolone (THK-523),[19] and (S)-1-(fluoro-[^{18}F])-3-((2-(6-(methylamino)pyridin-3-yl)quinolin-6-yl)oxy)propan-2-ol (THK-5351)[20] have reached clinical trials. These compounds could thus be useful for the differential diagnosis of neurological conditions in elderly patients, opening the door for new research to elucidate mechanisms of tau-mediated neurodegeneration, as well as tau-focused biomarkers and therapies.

3. Aβ and tau fluorescent ligands as the AD imaging agents of the future?

PET is currently one of the most popular clinical imaging techniques. However, its routine use for AD is unlikely due to its financial and technical burdens. For some countries, the high cost of PET scans prevents the clinical use of the above-mentioned PET tracers.[21] PET applications are further limited by the short half-lives of commonly used positron-emitting isotopes, which require the on-site synthesis of PET tracers and access to radiochemistry equipment and a cyclotron. In contrast, optical imaging is emerging as a cheaper and noninvasive in vivo tool with improved sensitivity and

resolution with respect to other routinely used methodologies, such as magnetic resonance imaging (MRI) and computed tomography (CT).[22] Optical imaging also holds potential for AD.[23]

In 2005, the oxazine derivative 4,8-dimethyl-3,8,9,10-tetrahydro-2*H*-xantheno[2,3-*b*:7,6-*b'*]bis([1,4]oxazine)-4-ium tetrafluoroborate (AOI987)[24] suggested for the first time the feasibility of noninvasively imaging cerebral β-amyloid deposits in living mice. Since then, the use of molecular fluorescence-based neuroimaging, particularly near-infrared (NIR) imaging, has rapidly gained momentum.[23] Molecular fluorescence-based neuroimaging avoids the technical difficulties of other modalities, such as the use of X-ray in CT and the facility requirements of PET probes.[22] Moreover, the first developed fluorescence methods, such as two-photon fluorescence microscopy, required craniotomy, cranial windows and skull thinning, as the resolution of optical imaging decreases with increasing depth of the source of fluorescence emission.[25] Conversely, fluorescence molecular tomography (FMT) has the potential to circumvent these limitations by exploiting different light sources and different theoretical models of image reconstruction.[25] Additionally, researchers are quickly overcoming these weaknesses by developing hybrid techniques, multiphoton microscopes,[26] and novel imaging agents.[27] For example, resolution and tissue penetration can be significantly improved by combining FMT with CT[28] and micro-CT,[29] or MRI[30] (see ref. [25] and [31] for a more detailed description). Additionally, the group of optical imaging pioneer Ntziachristos recently developed multispectral optoacoustic tomography (MSOT).[32] MSOT has allowed high-resolution visualization of glioblastoma deep inside tissues by the NIR fluorophore indocyanine green, through the intact mouse head.[33]

The impressive technological advances in fluorescent imaging have stimulated tremendous research efforts to develop novel agents with optimized biophysical and imaging properties. In particular, researchers are designing NIR fluorescent probes, since the light in the NIR spectral range (650–900 nm) minimizes autofluorescence (due to the fact that fluorescence from biosamples or living animals is normally below 600 nm) and increases penetration depth (due to the reduced absorbance from blood hemoglobin).[34]

Since 2005,[24] many fluorescent imaging probes have been published together with several more recent reviews of a wider scope.[35,36, 37] The interested reader is directed to these publications for information regarding the design and application of fluorescent probes for in vitro or in vivo imaging of Aβ plaques, both in AD mice and patients. Although at the moment NIR imaging is primarily limited to preclinical studies, those reported so far substantiate the potential of fluorescent imaging approach to advance personalized medicine in AD.[35] We are confident, that, even in the worst case, such studies will be very informative in a research context. We believe that they have already contributed to the preliminary development of the companion diagnostics and theranostics discussed herein.

4. The emergence of companion diagnostics in AD

Given the setbacks, lack of breakthroughs, and Phase III clinical trial flops that have characterized AD drug development in recent years, the pharmaceutical industry has been reconsidering how best to approach this pathology. One major switch is the goal of treating AD in its early stages, since it may be irreversible by the time clinical symptoms fully appear.[38] This approach considers early diagnosis, identification of the right target patients, and early intervention to be the key elements of a successful AD treatment.

Ideally, any pharmacological therapy decision should be timely and based on a comprehensive understanding of the disease biology and the drug mechanism of action. Although the end goal is not yet achieved, recent advances in AD molecular medicine and diagnostics have provided reasonable insights into how drug discovery, development, and pharmacotherapy could shift to a more rational biomarker-driven approach. As the field advances, pharmaceutical companies are moving to develop novel devices, such as CDx assays, to support AD drug research and therapy. Drawing together the definitions proposed by various regulatory authorities and pharmaceutical companies,[39,40] a CDx assay is a safe and effective in vitro diagnostic assay to measure a recognized indicator of either normal biological/pathogenic processes or pharmacological responses to a

therapeutic. As such, CDx provide biological and/or clinical data that allow researchers and clinicians to reach suitable and timely decisions. These include decisions concerning early drug development as well as the administration of personalized drug therapy during routine clinical care.[41]

Broadly, CDx have four potential applications: 1) selection of patients for a specific/targeted treatment; 2) staging markers to adapt the therapeutic strategy; 3) predictive markers for drug response (efficacy), resistance, and safety; 4) follow-up markers to check the therapeutic response (therapy monitoring) and to adjust therapy (alternative, dosing, duration) if required.[42]

CDx are changing the management of several diseases, as the old "population treatment" approach gives way to the groundbreaking concept of personalized medicine. In personalized medicine, subpopulations of patients are identified according to their "molecular profile", with certain subpopulations most likely to benefit from a targeted treatment and others at particularly high risk of experiencing side effects.[43] This strategy has been successfully applied to several therapeutic areas, providing excellent examples of CDx drug development, especially in oncology and virology.[40] AD drug research is moving in this direction, with big pharma seeking to develop reliable CDx assays for use in the design of clinical trials.

In this respect, both neuroimaging and cerebrospinal fluid (CSF) biomarkers ($A\beta_{42}$, total tau, and phosphorylated tau levels) have now been included in diagnostic criteria for AD and are regularly incorporated into clinical trials. The interested reader is directed to exhaustive reviews on the application of fluid biomarkers to the development of AD therapeutics,[44, 45] while examples of AD clinical programs that recently used the newly approved diagnostic PET tracer scans as CDx tools are discussed below (Table 1). For instance, **1** is currently being used in Phase III trials of the experimental anti-Aβ antibodies gantenerumab (Roche Holding AG)[46] and solanezumab (Eli Lilly)[47] as a pharmacodynamic measure of changes in brain amyloid load over time in mild and early-stage AD, respectively.

This is not the first time that Roche has used PET technology to support AD clinical investigations. In 2012, it launched a pioneering Phase II/III trial of gantenerumab (SCarlet RoAD) in prodromal (predementia) AD, using PET amyloid imaging to recruit participants (positive for amyloid PET) and to monitor Aβ brain level over time.[48] Although discontinued in 2014, this study was among the first examples of novel diagnostic criteria being incorporated into a large clinical trial. Moreover, the innovative screening process was successful in enrolling a homogenous population of early symptomatic AD patients, confirming the importance of CDx assays in designing robust and reliable trials.[48] Before SCarlet RoAD was discontinued, Roche's diagnostic division announced they would be developing a novel CDx assay for future use.[49]

Similarly, soon after its approval, **2** was licensed by GE Healthcare to Merck, Johnson & Johnson, and Eisai as a CDx for identifying appropriate patients for clinical trials and developing potential AD drugs.[50] In December 2012, Merck and GE Healthcare announced a collaboration to use **2** to support a Phase II/III trial of Merck's drug candidate **5** (MK-8931, verubecestat,[51] in Figure 2) in patients with prodromal AD.[52] **5** is a promising small molecule inhibitor of the β-secretase enzyme (BACE-1), which cleaves the amyloid precursor protein (APP), initiating the neurotoxic amyloid cascade.

In March 2013, Merck signed a deal with Luminex to develop a novel CDx assay to further support its ongoing **5** clinical program (Table 1).[53] Luminex will manage the development, regulatory submission, and commercialization of the candidate CDx device. This device will use Luminex's innovative xMAP technology to measure concentrations of two candidate biomarkers ($A\beta_{42}$ and tau) in CSF samples from patients with mild cognitive impairment (MCI). The candidate device will be evaluated as a tool for identifying subjects with MCI who have a higher risk of developing AD, as well as a tool for supporting patient selection and enrollment for the **5** clinical program.[53]

A substudy of the **5** study protocol will use the Luminex investigational tau/amyloid assay to evaluate response to treatment in CSF-positive participants (i.e. those with defined tau/amyloid-β_{42}

ratio in CSF) and to evaluate changes in CSF concentrations of Aβ-related peptides, total tau, and phosphorylated tau.[52]

The results of these trials should prove invaluable in answering important questions for BACE-1 inhibitor clinical development. For example, at what level should the BACE-1 inhibition be and at what AD stage should treatment be delivered for optimal efficacy?[54]

Although several PET tracers have been used to evaluate the efficacy of experimental AD drugs in human clinical trials, PET imaging is rarely used to monitor drug treatment in small animals. This is because of the complicated experimental procedures and data analysis in small animals, and the high cost of synthesis and scanning of PET probes combined with the use of radioactive material in the very early stages of drug discovery. In 2015, Moore and Ran proposed overcoming these challenges by using the curcumin analogue **6** (CRANAD-3)[55] as an NIR imaging probe to monitor the Aβ-lowering effectiveness of two different therapeutics in an AD mouse model (Figure 3). As already noted, NIR imaging is generally more suitable than PET imaging for animal studies, thanks to its lower cost, simpler operation, and easier data analysis. In previous studies, the same group showed that **11** (CRANAD-2 in Figure 7, see below),[56] a curcumin-based NIR imaging probe, displayed a significant increase in fluorescence intensity upon interacting with insoluble Aβ aggregates. However, **11** could not detect soluble Aβ species.[56] These are considered to be the most neurotoxic species and could thus potentially serve as biomarkers for the AD presymptomatic stage. On this basis, they synthesized a new derivative, **6**, by replacing the two phenyl rings of **11** with two pyridyls to establish hydrogen-bonding contacts with the engaged Aβ fragment (Figure 3). **6** displayed an emission peak at 730 nm and showed excellent fluorescence response toward soluble Aβ species. In vivo two-photon microscopic and NIR imaging also indicated that **6** is able to penetrate the BBB and is specific toward Aβ aggregates.[55] Based on this encouraging data, the authors investigated the ability of **6** to monitor the therapeutic effectiveness in APP/PS1 mice of two experimental drugs (Table 1), the BACE-1 inhibitor (4S)-4-[2,4-difluoro-5-(pyrimidin-5-yl)phenyl]-4-methyl-5,6-dihydro-4H-1,3-thiazin-2-amine (LY2811376), and the metal-mediated

$A\beta_{42}$ crosslinking inhibitor **7** (CRANAD-17), developed by the same group.[57] Structurally, LY2811376 is an amino thiazine derivative, whereas **7** is an imidazole-containing curcumin analogue.[57] **6** was successfully used to evaluate the therapeutic efficacy of both drug treatments in short and chronic mouse treatment studies, indicating that it could be considered the first NIR CDx suitable for monitoring the Aβ-lowering effectiveness of drugs. This seminal work also corroborated that fluorescence imaging agents could be valid alternatives to PET tracers as effective CDx.

The **6**/**7** pair is one of the first examples in the AD field of a drug-diagnostic codevelopment model (i.e. a diagnostic assay developed in conjunction with a targeted drug). The positive results suggest this strategy as an exceptionally strong investigational tool for AD drug research.

5. Theranostics and the prospects of personalized medicine in AD

In parallel to CDx, "theranostics" uses single chemical entities to deliver therapy and diagnosis simultaneously and is a more recent and equally innovative way of implementing translational medicine and personalized approaches.

The term was coined in 1998 by John Funkhouser[58] for the company Cardiovascular Diagnostics to describe a material that allows the combined diagnosis, treatment, and follow-up of a disease. Although its definition is still evolving, the following concepts, highlighted in the dedicated journal *Theranostics*, are illuminating: "*Theranostics is a concept that was originally raised to refer to the efforts of integrating imaging and therapy. As an emerging interdiscipline, it is related to but different from traditional imaging and therapeutics.*"[59] The theranostics concept is tightly intertwined with personalized medicine. It allows the selection of patient subpopulations, according to their "molecular profile" at a given time, who are most likely to benefit from a targeted therapy or at higher risk of adverse effects. Ideally, the simultaneous and fully integrated development of the drug and its diagnostic counterpart will distinguish theranostics from classical drug/diagnostic test combinations.[42]

Although still in its infancy, the field of theranostics has exploded over the past couple of years, with new agents reaching clinical trials at a rapid pace, especially in the field of cancer (see Kojima, 2015[60] for a recent review). The literature dealing with theranostics and AD has grown steadily. It is thought that these agents could potentially offer new hope to patients and real gains for pharmaceutical and diagnostic companies.[5, 61, 62,35, 63]

Analyzing the theranostic-related literature, the approaches reported to date involve two molecule classes: nanomedicines and small organic compounds. Oncology researchers have established a plethora of nanomedicines (including polymer–drug conjugates, liposomes, polymeric micelles, and inorganic nanoparticles) with distinctive chemical compositions and physical properties. Compared with traditional molecular-based contrast agents or therapeutic drugs, these new nanoplatforms are extremely versatile, allowing ultrasensitive imaging and therapy to be incorporated into one system in a highly integrated manner.[64-66] However, the development of theranostic nanomedicines for AD could be hindered by the limited ability of these molecules to cross the BBB and exploit CNS drug delivery, together with a somewhat unacceptable invasiveness, not compatible with a chronic treatment.[67] Despite this, several groups are actively engaged in the field and their efforts have recently been reviewed elsewhere.[68-71]

In principle, the above hurdles to the development of AD theranostics may be surmounted by the use of small molecules. But even here and despite tremendous potential, the rational design of small molecules with a predefined theranostic profile presents new and unexplored challenges. The discovery phase is likely to be particularly complex, as the medicinal chemistry community must consider new identification and optimization strategies.

From the examples already reported, we identify two main rational strategies: (i) linking structural elements from diagnostic and therapeutic agents to make a new conjugate molecule; (ii) the diagnostic and therapeutic moiety overlap or are highly integrated in a single chemical entity (Figure 4).

In the first approach, conjugated ligands contain the two starting units, one carrying the therapeutic properties and the other carrying the diagnostic properties, chemically connected by a spacer of a variable nature and length. In principle, the second approach has narrower applicability, since the basic prerequisite is that an imaging probe and a drug share a common structural scaffold. This design concept involves a single interacting protein that is both the therapeutic target and the biomarker. This could be the case for theranostic compounds developed to label and simultaneously inhibit aggregation of fibrils of pathological Aβ or tau. A particular strength in this scenario is that there can be overlap between the molecular properties responsible for recognizing and consequently inhibiting these amyloid proteins, and those responsible for their fluorescence imaging. Extended π-conjugated flat systems are considered important features for binding and interfering with amyloids.[72, 73] But they also result in increased electronic delocalization, which induces a small band gap between the excited and ground states, leading to an emission wavelength in the desired NIR spectral window.[74]

Of course, ligands designed by a conjugating approach are more likely to have a high molecular weight and less likely to have favorable BBB permeation, whereas overlapped compounds are likely to have lower molecular weight and, in principle, better drug-like properties. However, the linking strategy opens up possibilities to address PK issues with appropriate linker design. In principle, some of the problems associated with high hydrophobicity of the conjugate can be overcome by the use of PEG-containing hydrophilic linkers.[75]

Despite these challenges, an ever-increasing number of theranostics are being developed against AD. Notably, Moore and Ran's group has produced most of them. In addition to these elegant, rational approaches, the literature contains examples where the deliberate aim of generating a theranostic is not explicitly stated. However, the resulting small molecules have true theranostic potential because they can both image and inhibit Aβ peptides. In this respect, actual developments in the field may not be fully represented in the literature, since some authors use terms such as "multifunctional" or "bifunctional" rather than "theranostics".

In 2012, for example, Li, Yung and Wong[76] developed what they called "multifunctional fluorophores" able to directly image the dynamics of Aβ fibrillogenesis and inhibit its aggregation. They based their investigation upon previously identified carbazole-based cyanine fluorophores reported to bind to Aβ with concomitant strong fluorescence enhancement.[76] The carbazole scaffold was then variously functionalized to ameliorate key functional properties, such as Aβ-binding, cytotoxicity, and BBB permeability. Structure-activity relationship studies led to the discovery of the promising compound **8** (SLOH in Figure 5).[76] After the characterization of the intrinsic fluorescence properties, **8** demonstrated a strong and progressive increase in its fluorescence intensity, concomitant with a blue shift of the emission maximum, indicating a direct interaction with the Aβ peptide. Moreover, the fluorescence enhancement was much stronger for Aβ fibrils than for Aβ peptide. After validating **8**'s diagnostic capacity, the authors disclosed a therapeutic inhibitory effect on both the Aβ nucleation and elongation processes. The mechanism of the anti-amyloid profile was also confirmed by circular dichroism studies. Thus, after verifying that treatment with different concentrations of **8** was not toxic to SH-SY5Y cell line, the group investigated its effect on Aβ-induced cytotoxicity on the same cell line and on mouse primary cortical cells.[76] Significantly, **8** displayed a strong neuroprotective effect on both cell models. Finally, the authors demonstrated **8**'s abilities to pass the BBB in vivo and to ex vivo label Aβ plaques in APP/PS1 Tg mice.[76] Taken together, these results are suggestive of **8**'s theranostic potential. However, in vivo studies are needed to demonstrate the effective therapeutic and safety profile of **8**.

In 2013, members of our group developed the styrylquinoline **9** (**G8**),[77] one of the first small molecules to be intentionally proposed as an in vitro theranostic (Figure 6). Our inspiration was the finding that several styryl derivatives, designed to improve the pharmacokinetic properties of Congo red and ThT, had been successfully employed as amyloid imaging agents in vivo.[78] In addition to staining amyloid fibrils with high affinity, many of these molecules were found to block

their aggregation. We thus sought to develop a novel theranostic by exploiting a styrylquinoline core structure.

We selected the quinoline for its "privileged scaffold" nature, which, in principle, displays desirable pharmacokinetic properties and drug-likeness. Among other derivatives, we selected **9** and evaluated its theranostic profile for potentially diagnosing, delivering therapy, and monitoring responses in AD and prion diseases.[77] This double activity was proposed on the basis of the ever-increasing neuropathological commonalities found between these two devastating neurodegenerative diseases.[79] Thus, after verifying **9**'s encouraging fluorescence properties and its good BBB permeability, we separately evaluated its therapeutic and diagnostic activities. Inhibition towards $A\beta_{42}$ aggregation was assessed by the classical ThT-based fluorometric assay, where **9** significantly decreased the fluorescence signal, confirming its ability to act as a fibrilization inhibitor.[77] Moreover, when measuring the emission spectra in the absence and presence of Aβ aggregates, **9** showed a strong hypsochromic shift of the emission maximum, concomitant with a hyperchromic effect upon binding. This clearly highlighted its potential as an amyloid sensor. Finally, displacement studies suggested that **9** may have a primary high-affinity binding site distinct from ThT and a secondary low-affinity binding site in common with ThT. As expected, a similar anti-aggregating activity was observed when **9** was tested against prion protein in an in vitro amyloid seeding assay. On the basis of these promising outcomes, we evaluated the global theranostic potential of **9** in the ScGT1 cellular model of prion diseases. After excluding that treatment of **9** was toxic to the cells, we evaluated its inhibitory activity against prion replication, and found a relevant submicromolar antiprion effect.[77] To corroborate the labeling of fibrillar aggregates in living cells, fluorescent staining with **9** was carried out using the same prion cell model. Treatment with **9** allowed us to visualize a number of localized fluorescent spots in the infected cells examined by fluorescent microscopy. Even more significant in terms of selectivity, we observed no spots in the control uninfected cells. Thus, although our study is limited to a cellular context and does not definitively prove **9**'s theranostic potential, it offers a promising

starting point for developing novel theranostic tools to in vivo diagnose, deliver targeted therapy, and monitor responses to therapy in both AD and prion diseases. As regard as toxicity, although we did not find any sign of cytotoxicity up to 10 µM, compound **9** presents both aniline as well as a styryl olefin, two moieties known to bring safety issues in drug candidates.

The curcumin analogue **10** (CRANAD-28 in Figure 7), proposed in 2014 by Moore and Ran,[80] is another example of a rationally designed theranostic agent. Moore and Ran began with a previously developed curcumin analogue (**11** in Figure 7) that could detect soluble and insoluble Aβ species,[56] but was endowed with low quantum yield (QY). To overcome this, the authors proposed replacing its phenyl rings with pyrazoles.[80] They reasoned that the inductive electron-withdrawing effect of the nitrogen of pyrazole could lead to a low tendency of electron delocalization in the system, which would decrease tautomerization, increasing the QY. This idea was supported by their previous studies, which showed that imidazole curcumin analogues effectively interfere with the coordination of Cu^{2+} and thus attenuate the pathological crosslinking of Aβ that is mediated by this metal.[56]

On these logical foundations, they synthesized **12** (CRANAD-44 in Figure 7)[80] so that the pyrazoles could interfere with Cu^{2+} coordination while simultaneously reducing tautomerization and increasing QY. To improve **12**'s properties, they developed the corresponding N-1 phenyl derivative **10**, which underwent extensive investigation for its theranostic potential in comparison to **12**.[80]

First, they characterized the fluorescence properties and QY of both molecules. As expected, the QY of **10** was higher than that of **12**.[80] They then tested the ability of **10** to interact with Aβ (Figure 7). Interestingly, **10**'s fluorescence intensity was quenched by Aβ, indicating a direct interaction. Staining APP/PS1 mouse brain slices with both molecules showed that **10** provided an excellent contrast for Aβ plaques, while **12** did not, suggesting that the phenyl rings at the N-1 position successfully enhance Aβ binding in accordance with **10**'s rational design. Finally, after assessing the BBB permeation, the authors validated the capability of **10** for the in vivo two-photon imaging

of APP/PS1 mice.[80] As anticipated, Aβ plaques were clearly labeled and cerebral amyloid angiopathies (CAAs) were also highlighted. To definitively validate **10**'s theranostic potential, the authors sought to verify the ability of the pyrazole rings to coordinate Cu^{2+} and thus exert the desired therapeutic effect.[80] Western blotting analysis showed that, unlike **12**, **10** significantly inhibited Cu^{2+}-induced crosslinking of Aβ. Accordingly, **10**'s great significance for theranostics is that, despite emitting outside the intended NIR wavelength region (λ_{em} > 578), it has provided the first in vivo proof of principle in an AD mouse model. Despite CRANAD derivatives **10**-**12** are extremely promising starting point for theranostic development, it should be pointed out that curcumin analogues present possible Michael acceptor moieties. To best of our knowledge, possible safety liabilities linked to this functionality have not been addressed in the previously mentioned papers.

In 2014, Moore and Ran proposed another enlightening approach to the development of Aβ-directed theranostics, by exploiting a linking approach.[81] It is widely known that crown ethers form stable complexes with protonated amines through hydrogen-bond formation. In particular, positively charged Aβ fibrils are highly cytotoxic, whereas neutralizing the charges reduces neuronal toxicity. The authors hypothesized that crown ethers could form noncovalent complexes with Aβ peptides with positively charged basic amino acids such as K16 and K28,[81] which form intrapeptide salt bridges with D23 leading to the stabilization of the misfolded peptides.[82] Their working hypothesis was that breaking down these salt bridges could attenuate the Aβ aggregation process. As a preliminary test of their hypothesis, they assessed the anti-aggregating ability of a 12-crown-4 ether, rationally selected for its low molecular weight, its effective ability to form complexes with charged amino acids, and its insignificant disturbance of physiological ion homeostasis. Both ThT testing and dot blotting indicated that this molecule could efficiently inhibit Aβ aggregation. To achieve a selective targeting, the authors therefore linked the 12-crown-4 ether at the amino group of **4**, leading to the conjugate **13** (PiB-C in Figure 8).[81] Encouragingly, **13** was

more potent than **4**, indicating that the crown-ether moiety markedly contributed to the inhibitory effect against Aβ aggregation.

The authors then treated SH-SY5Y cells with $A\beta_{42}$ in the absence or presence of 12-crown-4 ether and **13**.[81] Both molecules lowered the toxicity of $A\beta_{42}$, while **4** did not, indicating that **13**'s neuroprotective ability was not due to the presence of the **4** moiety. Finally, since **13** efficiently crossed the BBB, the authors tested its ability to specifically label Aβ plaques in vitro and in vivo, using two-photon imaging. As expected, **13** could clearly image plaques in APP/PS1 mouse brains in vitro and, more importantly, in vivo.[81] Moreover, **13** could also efficiently label CAAs in a similar manner as **4**.

14 (TBT in Figure 9), developed in 2015 by Yao and Wang,[83] is the most recently proposed theranostic small molecule. They followed a very similar approach to that reported by Moore and Ran for **13**. Here, the inspiration was the therapeutic potential of metal chelators to reduce metal-induced Aβ aggregation and neurotoxicity in AD.[84] The authors thus developed a novel fluorescent chelator (**14**) as a dual functional probe and disaggregating agent for Aβ aggregation induced by Zn^{2+} and Cu^{2+}.[83] Following a linking approach very similar to that used for **13**, they designed **14**, which comprises a metal-chelating 1,4,7,10-tetraazacyclododecane (cyclen) group and, again, the **4** Aβ-recognizing scaffold.

As a positive feature of the chelating profile, **14** can tightly bind to Zn^{2+} or Cu^{2+}, while exhibiting high selectivity over other biologically relevant metal ions. In terms of its anti-amyloid profile, **14**'s high binding affinity for Aβ aggregates was disclosed using the ThT fluorescence competition assay.[83] The authors found that the high affinity of **14** for Zn^{2+}-$A\beta_{40}$ aggregates results from a synergistic effect of the two functional moieties. Additionally, molecular docking analysis showed that **14** competes with ThT due to the overlapping binding sites in Aβ, and captures metal ions from metal-Aβ species. After confirming that **14** specifically inhibits Zn^{2+}- and Cu^{2+}-induced $A\beta_{40}$ aggregation over metal-free $A\beta_{40}$ aggregation in vitro, the authors monitored the disaggregation of $A\beta_{40}$ aggregates in brain homogenates of APPswe/PSEN1 Tg mice.[83] These results, together with

14's specificity, neuroprotection, and efficient BBB permeability, highlight this fluorescent chelator's potential as a theranostic tool for AD.

Conclusion and perspectives

"Few candidates, frequent failures" is the best description for the current status of AD drug development. Summing up what we have discussed in this Miniperspective, CDx and theranostics emerge as powerful tools to change the status quo, by potentially de-risking development projects, shortening development timelines, allowing faster new product approval, and ultimately generating revenue streams.

CDx are becoming a well-established approach for many major diseases, particularly cancer. Their underlying concept is that such major diseases are immensely heterogeneous, and the existing treatments are effective only for limited patient populations and at particular stages of disease development. Thus, a close marriage of diagnosis and therapeutics could better tailor therapeutic practices to individuals, offering improved prognoses.[85] This also applies to AD, for which, more crucially, there are currently no disease-modifying treatments.

In parallel to CDx, the development of theranostics is growing in feasibility and attractiveness as a strategy. In the CDx approaches discussed above, imaging probes and drugs are pursued separately, which is resource-hungry, time-consuming, and costly. Moreover, in some cases, their use has led to mixed results in clinical trials. Conversely, developing single agents with both imaging and therapy properties could be inherently simpler and more economical, expediting the drug discovery process. More importantly, theranostics could overcome a problem related to the use of biomarkers and CDx and identified by the Alzheimer's Association's Research Roundtable.[86] Even if a biomarker is relevant to AD and can be monitored by a corresponding imaging agent, the imaging agent's clinical utility as a CDx is not guaranteed. For example, both bapineuzumab and solanezumab are monoclonal antibodies directed against the $A\beta_{42}$ peptide.[86] However, they show different relations between the cerebral amyloid burden imaged by **4** PET scanning and the clinical

outcome. This may be due to differences in the binding properties of these two antibodies. While bapineuzumab has a higher affinity for amyloid plaques than soluble Aβ, solanezumab showed preferential binding to soluble Aβ but not to aggregated Aβ. This indicates that not all biomarkers, and consequently CDx imaging agents, are interchangeable for use in all trials. However, "overlapped" theranostics overcomes this problem, since the target for therapy and the biomarker for disease-monitoring coincide. Recent examples of theranostics' potential are highly gratifying, and augur well for their future applicability to AD. However, their full value will not be realized until their diagnostic and therapeutic applications are deployed at the clinical level.

Can researchers take theranostics to clinical trials? It will require a blend of pragmatism, utopia, and cooperation. Identifying future paths to radical improvement will require a rigorous, detailed understanding of the most critical challenges (pharmaceutical, clinical, regulatory). At the same time, the theranostics concept demands a drastic rethink of the way we develop drugs and diagnostics. Collaboration between pharmaceutical and diagnostic companies will be crucial to fostering this innovation. Thus, we could be on the cusp of curing AD, but bold and sustained action is required from many actors to fully exploit these scientific opportunities. Given the current unmet medical needs and the advantages if successful, this bold step is warranted.

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ABBREVIATIONS

AD, Alzheimer's disease; Aβ, amyloid-β; FDA, Food and Drug Administration; CNS, central nervous system; BBB, blood-brain barrier; PET, positron emission tomography; CDx, companion diagnostics; ThT, thioflavin T; MRI, magnetic resonance imaging; CT, computed tomography; NIR,

near-infrared; FMT, fluorescence molecular tomography; MSOT, multispectral optoacoustic tomography; QY, quantum yield; BACE-1, β-secretase enzyme; APP, amyloid precursor protein; MCI, mild cognitive impairment; CSF, cerebrospinal fluid; CAAs, cerebral amyloid angiopathies.

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Biographies

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Annachiara Gandini received her Master's degree in Chemistry and Pharmaceutical Technology in 2014 from the University of Bologna. Currently, she is a doctoral student under the supervision of Prof. M. L. Bolognesi at the University of Bologna. Her research project concerns the development of small molecules as theranostics for neurodegenerative diseases, in collaboration with Prof. G. Legname of SISSA, Trieste.

Federica Prati received her Master's degree in Chemistry and Pharmaceutical Technology in 2010 from the University of Bologna. She obtained a PhD in Drug Discovery in 2014 from the University of Genoa, working on a project in collaboration with the Italian Institute of Technology to develop novel lead compounds against Alzheimer's disease, under the supervision of Prof. M. L. Bolognesi. In 2013, as part of her PhD program, she was a visiting scholar in Prof. I. H. Gilbert's group at the

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Elisa Uliassi received her Master's degree in Chemistry and Pharmaceutical Technology in 2012 from the University of Bologna. She is currently a temporary research fellow in the Bolognesi lab, admitted to the final oral defense by the PhD board in Chemistry at the same institution. From 2014 to 2015, as part of her PhD program, she was a visiting PhD student in Dr. Kenneth A. Jacobson's group at the National Institutes of Health (Bethesda). Her research work focuses on developing small molecules as chemical probes for stem cell applications in the field of Alzheimer's disease.

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Table 1. CDx assays used in current AD clinical trials and preclinical research.

CDx/Experimental drug	In vivo imaging	Biomarker	Drug MOA	Reference
[^{18}F]florbetapir/gantenerumab	PET	Aβ	anti-Aβ antibody	46
[^{18}F]florbetapir/solanezumab	PET	Aβ	anti-Aβ antibody	47
[^{18}F]flutemetamol/MK-8931	PET	Aβ	BACE-1 inhibitor	52
Luminex device/MK-8931	Luminex's xMAP®	$Aβ_{42}$ and tau	BACE-1 inhibitor	53
CRANAD-3/LY2811376	NIR	Aβ	BACE-1 inhibitor	55
CRANAD-3/CRANAD-17	NIR	Aβ	metal-mediated $Aβ_{42}$ crosslinking inhibitor	55

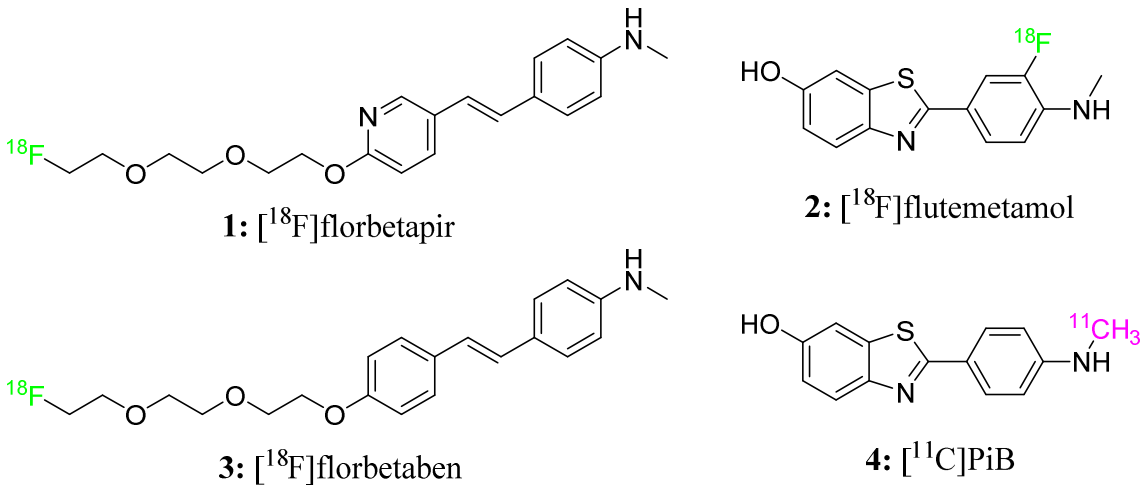
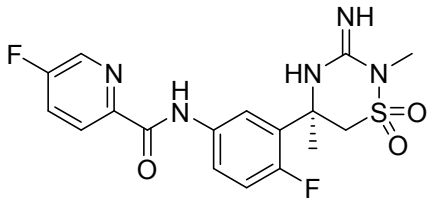


Figure 1. Amyloid PET imaging agents used in clinics (**1-3**) or in vivo human studies (**4**).



5: MK-8931

Figure 2. Chemical structure of BACE-1 inhibitor **5**.

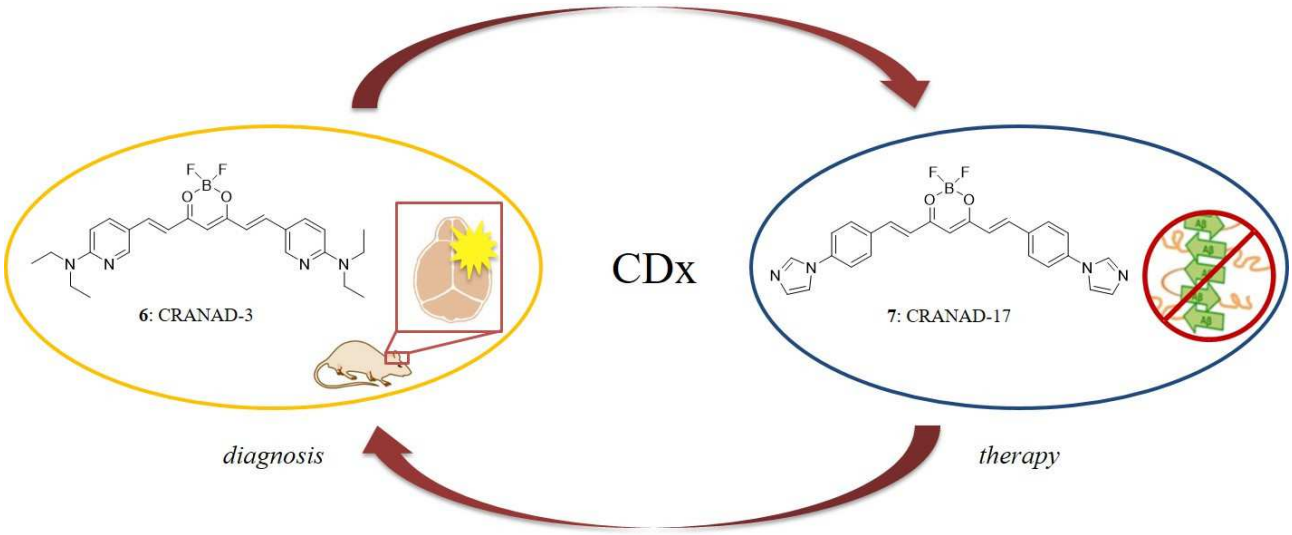


Figure 3. Development of the first fluorescent CDx validated in an AD mouse model.

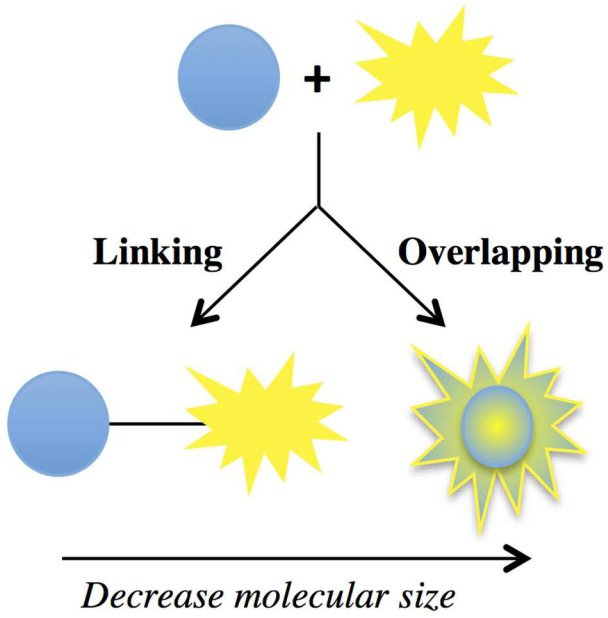


Figure 4. Design strategy of theranostics.

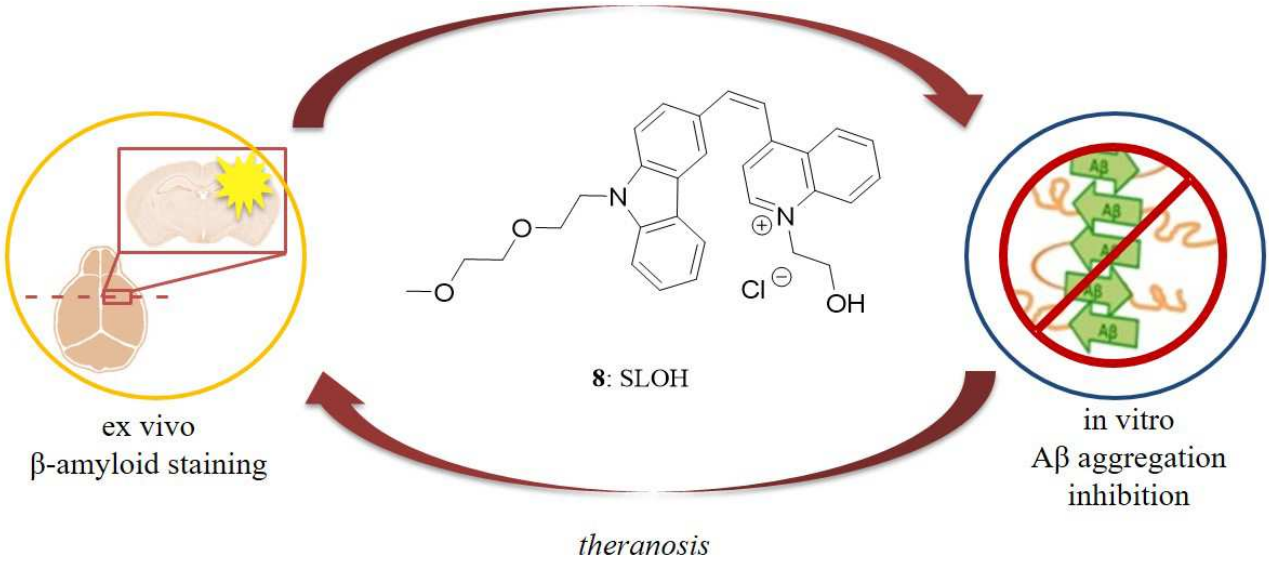


Figure 5. Theranostic profile of **8**.

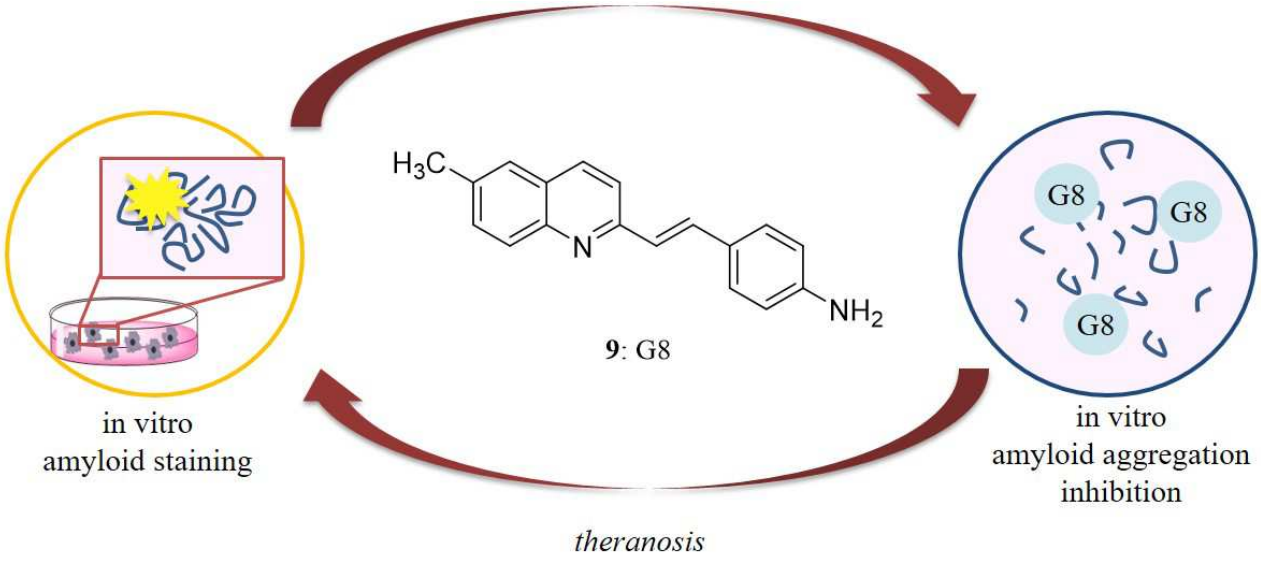


Figure 6. Theranostic profile of **9**.

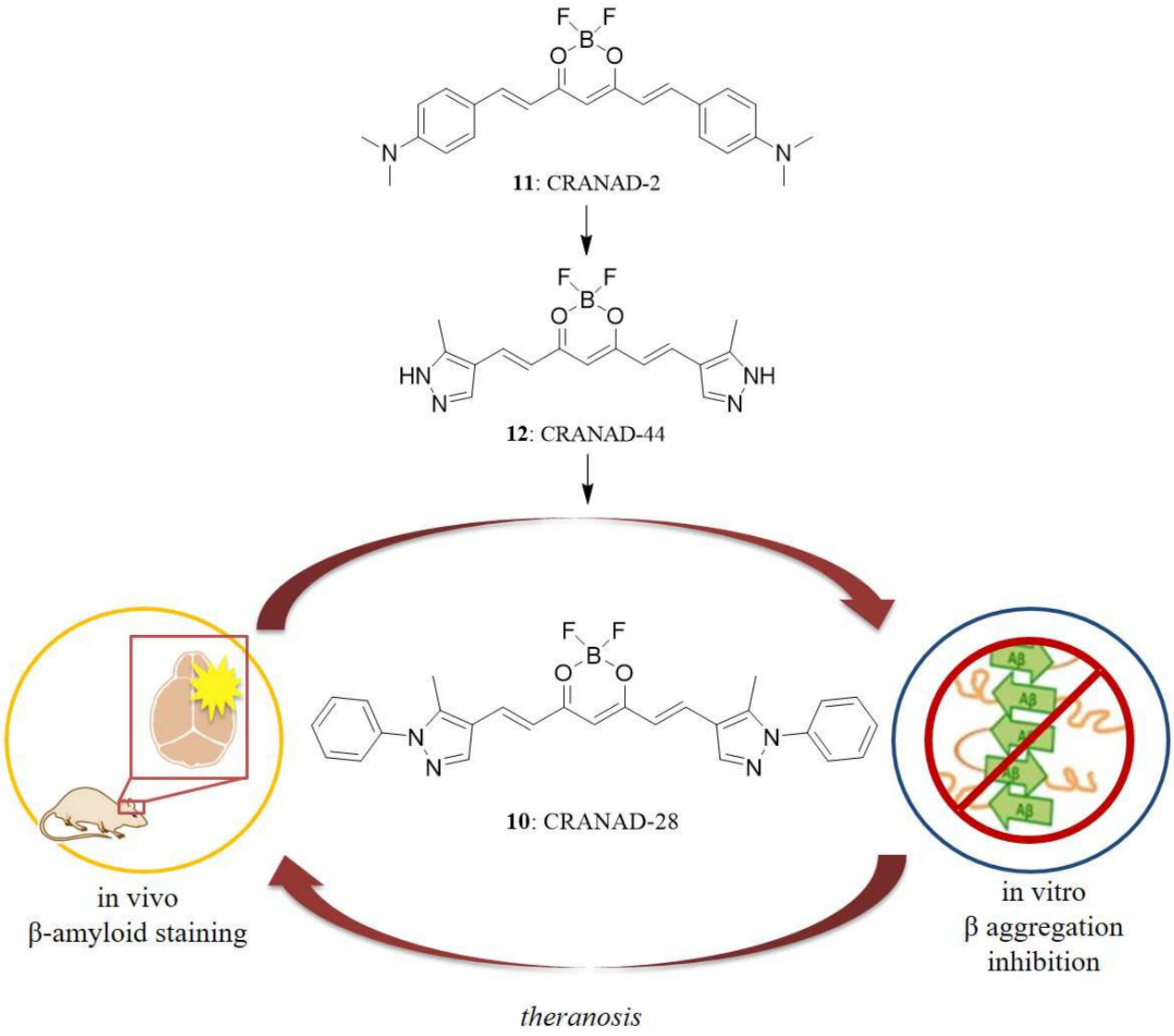


Figure 7. Rational pathway to curcumin-based theranostic **10** and its theranostic profile.

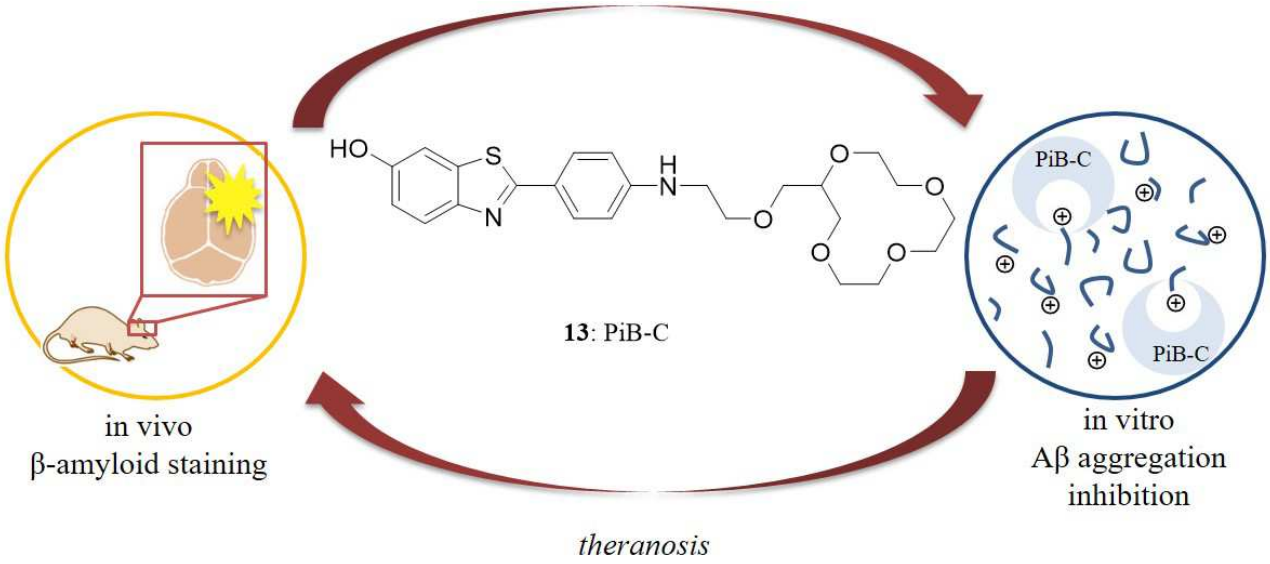


Figure 8. Theranostic profile of **13**.

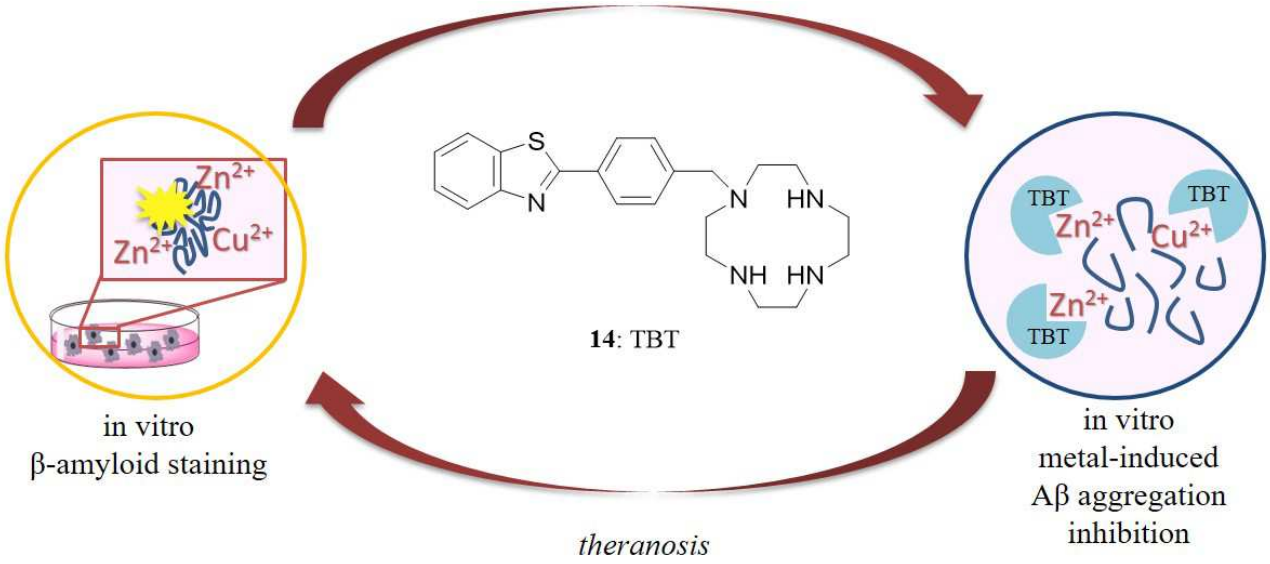


Figure 9. Theranostic profile of **14**.

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